AD-A283 970

REPORT DOCUMENTATION PAGE

OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1994	Final, N/A

4. TITLE AND SUBTITLE
Nitrogen dioxide-induced acute lung injury in sheep

5. FUNDING NUMBERS

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER
N/A

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Department of Respiratory Research (SGRD-UWH-E)
Division of Medicine
Walter Reed Army Institute of Research
Washington, DC 20307-5100

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
N/A

11. SUPPLEMENTARY NOTES
N/A

DTIC ELECTE AUG 30

12a. DISTRIBUTION/AVAILABILITY STATEMENT
UNCLASSIFIED/UNLIMITED

12b. DISTRIBUTION CODE
N/A

13. ABSTRACT (Maximum 200 words)

Lung mechanics, hemodynamics and blood chemistries were assessed in sheep before, and up to 24 h after, a 15-20 min exposure to either air (control) or 500 ppm nitrogen dioxide (NO_2). Nose-only (NO) and lung-only (LO) routes of exposure were compared for effects on NO_2 pathogenesis. Bronchoalveolar lavage fluids (BALF) from exposed sheep were analyzed for biochemical/cellular signs of NO_2 insult. The influence of breathing pattern on NO_2 dose was also assessed. NO_2 exposure of intubated sheep (LO exposure) was marked by a statistically significant blood methemoglobin increase. The exposure induced an immediate tidal volume decrease, and an increase in both breathing rate and inspired minute ventilation. Pulmonary function, indexed by lung resistance (R_L) and dynamic lung compliance (C_{dyn}), progressively deteriorated after exposure. Maximal R_L and C_{dyn} changes occurred at 24 h post exposure, concomitant with arterial hypoxemia. BALF epithelial cell number and total protein significantly increased while macrophage number significantly decreased within the 24-h post-exposure period. Examination of lung tissue 24 after NO_2 revealed patchy edema, mild hemorrhage and polymorphonuclear and mononuclear leukocyte infiltration. The NO_2 toxicologic profile was significantly attenuated when sheep were exposed to the gas through a face mask (NO exposure). Respiratory pattern was not significantly altered, lung mechanics changes were minimal, hypoxemia did not occur, and evidence of exudation was not apparent in NO, NO_2-exposed sheep. The responses of sheep to high-level NO_2 support the concept of NO_2 size-dependent species sensitivity. Moreover, when minute ventilation was used as a dose-determinant, a linear relationship between NO_2 dose and lung resistance was found.

14. SUBJECT TERMS	15. NUMBER OF PAGES
sheep, nitrogen dioxide, lung mechanics, hemodynamics, bronchoalveolar lavage	21
DTIC QUALITY INSPECTED 1	16. PRICE CODE N/A

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED

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Toxicology 89 (1994) 279–300

TOXICOLOGY

Nitrogen dioxide-induced acute lung injury in sheep

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(Received 29 January 1993; revision received 15 October 1993; accepted 28 December 1993)

Abstract

Lung mechanics, hemodynamics and blood chemistries were assessed in sheep (*Ovis aries*) before, and up to 24 h following, a 15–20 min exposure to either air (control) or approximately 500 ppm nitrogen dioxide (NO_2). Histopathologic examinations of lung tissues were performed 24 h after exposure. Nose-only and lung-only routes of exposure were compared for effects on NO_2 pathogenesis. Bronchoalveolar lavage fluids from air- and NO_2-exposed sheep were analyzed for biochemical and cellular signs of NO_2 insult. The influence of breathing pattern on NO_2 dose was also assessed. Five hundred ppm NO_2 exposure of intubated sheep (lung-only exposure) was marked by a statistically significant, albeit small, blood methemoglobin increase. The exposure induced an immediate tidal volume decrease, and an increase in both breathing rate and inspired minute ventilation. Pulmonary function, indexed by lung resistance and dynamic lung compliance, progressively deteriorated after exposure. Maximal lung resistance and dynamic lung compliance changes occurred at 24 h post exposure, concomitant with arterial hypoxemia. Bronchoalveolar lavage fluid epithelial cell number and total protein were significantly increased while macrophage number was significantly decreased within the 24 h post-exposure period. Histopathologic examination of lung tissue 24 h after NO_2 revealed patchy edema, mild hemorrhage and polymorphonuclear and mononuclear leukocyte infiltration. The NO_2 toxicologic profile was significantly attenuated when sheep were exposed to the gas through a face mask (nose-only exposure). Respiratory pattern was not significantly altered, lung mechanics changes were minimal, hypoxemia did not occur, and pathologic evidence of exudation was not apparent in nose-only, NO_2-exposed sheep. The qualitative responses of this large animal species to high-level NO_2 supports the concept of size dependent species sensitivity to NO_2. In addition, when inspired minute ventilation was used as a dose-determinant, a linear relationship between NO_2 dose and lung resistance was found. The importance of these findings, NO_2 dose-determinants, and the utility of sheep as a large animal inhalation model are discussed.

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Disclaimer: The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense. In conducting the research described in this report, the investigators adhered to the standards set forth in the 'Guide for Care and Use of Laboratory Animals' (NIH Publication 85–23) as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and in the Animal Welfare Acts (US PL 89–544; 91–579; 94–279).

0300-483X/94/$07.00 © 1994 Elsevier Science Ireland Ltd. All rights reserved.
SSDI 0300-483X(94)02802-2

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Key words: Sheep; Nitrogen dioxide; Lung mechanics; Hemodynamics; Bronchoalveolar lavage

1. Introduction

Nitrogen dioxide (NO_2) generated from the burning of nitrogen-containing propellants or munitions, or nitrogen fixation during intense combustion, presents a formidable occupational concern for soldiers in crew compartments of combat vehicles. This battlefield hazard is similar to that observed for the civilian occupation, silo-filling: short duration exposure to high-concentration NO_2 in confined spaces (Lowry and Schuman, 1956; Zwemer et al., 1992). A dissimilarity between these civilian and military workplace hazards is that escape to a non-hostile environment may not be immediately achievable on the battlefield. Therefore, it is important to understand what risk a short-duration exposure to high-concentration NO_2 might impart to a soldier and whether that individual would have the capacity to complete a mission, perform physical tasks, or advance to a safe position. Certainly, numerous factors are to be considered in addressing this problem. However, it is difficult to estimate functional incapacitation or life-threatening levels for humans, based on available literature. Anecdotal reports of human accidental exposure often lack critical information on exposure conditions, such as gas concentration or exposure duration. Moreover, regarding experimental animal studies, a size-dependant species sensitivity appears to exist (Carson et al., 1962; Book, 1982) and generally, there is a paucity of information on functional incapacitation following high-concentration NO_2 in large animal species. Large animal studies of high-level NO_2 have largely focused on mortality or pathology. In addition, NO_2 dose-response assessments in whole-animal studies have generally limited the dose characterizations to the exposure duration and gas concentration variables, with little attention given to the influence of breathing pattern on dose.

Our laboratory has studied pulmonary NO_2 toxicity in awake sheep. This species has been successfully used to study the biological hazards of other airborne environmental pollutants including smoke, ozone, sulfur dioxide, diesel exhaust, and carbon monoxide (Loick et al., 1992; Schelegle et al., 1990; Abraham et al., 1980a, 1981; Wang and Yang, 1990). Furthermore, a substantial database of sheep physiology exists (Hecker, 1983). In the experiments described herein, lung structure and function, and blood and bronchoalveolar lavage constituents were examined for NO_2-induced changes. The effects of nose-breathing and simulated mouth-breathing on NO_2 pathogenesis were also examined. Attention was given to fundamental factors of exposure which could influence the expression of pulmonary NO_2 toxicity, and NO_2 dose estimations were calculated.

2. Methods

2.1. Animal preparation

Twenty-seven respiratory disease-free crossbred ewes (35–50 kg) were obtained from a commercial supplier (Ovine Technologies, Inc., New Hope, PA). The sheep were prepared with a chronic carotid artery loop (Bone et al., 1962) 1 month prior to study. They were fasted 24 h prior to experimentation but allowed water ad libitum.

On the day of an experiment, a sheep was suspended in a sling attached to the inside of a large-animal metabolism cart. This procedure minimally restrained the sheep and immobilized the head and neck region while imparting minimal discomfort (Coulson et al., 1989). Ten percent lidocaine spray (Xylocaine, Astra Pharmaceutical Products, Inc., West Roxbury, MA) was used to topically anesthetize the nasal passages. A 7.5 mm i.d. foam-cuffed nasotracheal tube (Bivona, Inc., Gary, IN) was lubricated with 2% viscous lidocaine (Barre-National, Inc., Baltimore, MD). A bronchoscope was used to transnasally pass the nasotracheal tube below the larynx for pulmonary mechanics and respiratory measurements.

The sheep was surgically prepared with venous and arterial catheters for blood sampling, and cardiovascular and core temperature measurements. Catheters were aseptically placed using kits (Accuguide, Burron Medical, Inc., Bethlehem, PA) after local anesthesia with 2% lidocaine (Xylocaine, Astra Pharmaceutical Products, Inc., West Roxbury, MA). A percutaneous approach to the carotid artery in the loop was made and a 14-gauge lateral hole catheter was introduced and sutured into place. Similarly, an 8-French catheter with hemostasis valve was introduced into the jugular vein, through which a 7-French thermodilution catheter was inserted and passed through the right heart into the pulmonary artery.

2.2. Pulmonary mechanics and respiration

A 4-French catheter pressure transducer (model PR-219, Millar Micro-Tip, Millar Instruments, Inc., Houston, TX) was inserted through a nasotracheal tube side port and advanced to the distal end of the tube for trachea lateral pressure determinations. The non-intubated nasal passage was topically anesthetized and a second catheter transducer (8-French, model PR-346, Millar Micro-Tip, Millar Instruments, Inc., Houston, TX) was passed transnasally to the esophagus for pleural pressure estimation. The pressure tracing from the esophageal transducer was accepted as pleural pressure when signals showed negative deflection upon inspiration and when clearly defined cardiac oscillations were observed in the waveforms. Transpulmonary pressure was calculated as the difference between trachea lateral pressure and pleural pressure. A heated pneumotachometer (Series 3700, Hans-Rudolf, Inc., Kansas City, KS) was connected to the proximal

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end of the nasotracheal tube. Tidal volumes were derived from integrated flow signals. Isovolume technique was used to calculate lung resistance (R_L) from flow, volume and transpulmonary pressure signals while dynamic lung compliance (C_{dyn}) was derived from transpulmonary pressure and tidal volume signals at points of zero airflow. Inspired minute ventilation was calculated as the product of mean tidal volume and breathing rate. However, during exposure, inspired minute ventilation was calculated from inspired air volume, over time, measured with a turbine flowmeter (model VMM-2A, Sensormedics Corp., Anaheim, CA). The flowmeter was positioned in-line between the test gas and the animal.

2.3. Hemodynamics

Fluid-filled strain gauges (model P23XL, Spectramed Inc., Oxnard, CA) were connected to the thermodilution and arterial catheters to monitor heart rate, and carotid artery (P_a), right atrium (P_{ra}), pulmonary artery (P_{pa}) and pulmonary artery wedge (P_{pw}) pressures. Catheter positioning was verified by characteristic blood pressure waveforms. The thermodilution catheter was positioned in the pulmonary artery such that a wedge pressure (pulmonary artery occlusion pressure) was obtained upon balloon inflation. A computer (model SP1445, Spectramed, Inc., Oxnard, CA) was used to measure cardiac output ($\dot{Q}_T$) by thermodilution technique. A minimum of three measurements were made per sampling time and the values averaged. The computer also displayed a core temperature measurement. Systemic vascular resistance was calculated as $(P_a - P_{ra})/\dot{Q}_T$ and pulmonary vascular resistance as $(P_{pa} - P_{pw})/\dot{Q}_T$. Hemodynamic and respiratory variables were collected using a direct writing recorder (model 2800S, Gould, Inc., Cleveland, OH).

2.4. Bronchoalveolar lavage (BAL)

A fiber-optic bronchoscope (Pentax model FB-PP10, Precision Instrument Corporation, Orangeburg, NY) with 90-cm insertion tube was modified with a small piece of silastic tubing to increase the outer diameter of the insertion tube distal tip from 3 mm to 6 mm. Bronchoalveolar lavages were performed according to accepted procedure (Crystal et al., 1986). Lavages were performed on the right lung. The left lung served as negative control. Prior to lavage, the nasal passages and large lower airways were topically anesthetized with 2% lidocaine to reduce discomfort and coughing. Subsequently, the bronchoscope tip was advanced to the right caudal lobe bronchus until it was wedged in a fifth-generation subsegmental bronchus. Different ventral segmental bronchi of the right caudal lobe were sampled. The first ventral segment was used for lavages performed at baseline (−2 weeks) and immediately post exposure. The second and third ventral segments were used

for lavages at the 6- and 24-h sampling times, respectively. Six 30-ml and one 20-ml aliquots (total 200 ml) of sterile physiologic saline (39°C) were slowly instilled and gently aspirated, using approximately a 5-s dwell time. The aspirated fluid volume was recorded. The BAL fluids (BALF) were pooled and immediately cooled to 4°C. A cell count was taken using a Neubauer hemocytometer. A cell differential was performed on a small sample of BALF using a cell preparation system (Cytospin 3, Shandon Scientific Ltd., Astmoor, UK). A minimum of 300 cells per differential were counted by a pathologist who had no prior knowledge of the treatment groups. Epithelial cells were included in the count. Epithelial cells were characterized by columnar to tall cuboidal shape, frequent appearance of cilia, and oval nucleus with basophilic cytoplasm. Characteristic appearance of monocytes (ground-glass, lightly basophilic cytoplasm which usually contained vacuoles) aided in differentiating immature macrophages, migrating from the blood, from rounded cuboidal epithelial cells. The remainder of BALF was centrifuged (400 × *g*, 10 min, 4°C). The supernatant was acidified (10% acetic acid) to arrest phospholipid and eicosanoid metabolism, and refrigerated (−80°C) until further biochemical analyses could be performed. Total BALF protein was determined spectrophotometrically using a modified Lowry method which required deoxycholate to solubilize protein and remove phospholipids, which interfere with the protein measurement (Bollag and Edelstein, 1991).

2.5. Other measurements

Arterial and mixed-venous blood samples were anaerobically collected for blood gas analyses. The samples were evaluated with both an automated pH/blood gas analyzer (model I.L. 1306, Instrumentation Laboratories, Lexington, MA) and an oximeter (model OSM 3, Radiometer, Copenhagen, Denmark). The analyzers measured whole blood pH, oxygen and carbon dioxide partial pressures, plasma bicarbonate, total hemoglobin and methemoglobin. Hematocrit was determined by centrifugation. Oxygen consumption was calculated as the product of the arterio-venous oxygen content difference and $\dot{Q}_T$.

2.6. Pathology

Following a diazepam/ketamine overdose, sheep were euthanized by exsanguination. The lungs were first examined for gross pathological lesions and tissue specimens were prepared for microscopic evaluation. In nose-only and lung-only experiments, the lungs were initially perfused in situ with 0.9% saline by low-pressure (<25 mmHg) perfusion through the abdominal vena cava. Subsequently, the lungs were pre-fixed by perfusion with 4% formaldehyde:1% glutaraldehyde phosphate buffer solution. The lungs were removed

en bloc and tissue samples obtained. Tissue samples were fixed, processed, and stained with hematoxylin and eosin. Stained preparations were examined by light microscopy for lesions and signs of inflammation. Lung tissue from three air exposed sheep served as negative control.

Sheep lungs from BAL experiments were also examined for gross and histopathologic lesions. Following euthanasia, the left middle lobe was excised and tissue samples were subjected to gravimetric analysis. A clamp was placed on the remaining stump. The lungs were pre-fixed by intratracheal instillation of 4% formaldehyde:1% glutaraldehyde phosphate buffer, at a perfusion pressure that did not exceed 30 cm H_2O. Lung tissue samples were removed from 20 pre-determined sites, which included proximal and distal portions of all lobes. A gross pathological and histological scoring scheme was used by a pathologist who had no prior knowledge of the treatment groups.

2.7. Exposures

Sheep were exposed to either medical-grade air (control) or ≈ 500 ppm NO_2 in air (Matheson Gas Products, Baltimore, MD). Measured NO_2 concentrations for the nose-only, lung-only, and BAL experiments were 514 ± 31, 492 ± 34, and 522 ± 5 ppm, respectively. Exposure durations were 15 min for the nose-only vs. lung-only experiments, and 20 min for the BAL experiments. The sheep breathed the test gas ad libitum through a two-way non-rebreathing valve from a dynamic exposure system. All exposures in BAL experiments, and lung-only exposures, were administered through the nasotracheal tube. A plastic canine anesthesia mask with a rubber diaphragm (A.J. Buck & Sons, Inc., Cockeysville, MD) was modified with a 3-cm i.d. inlet and used for the nose-only exposures. The gas delivery system was composed of glass, teflon or teflon-conditioned components. The test gas reached 99% of the nominal concentration at the nasotracheal tube/mask inlet within 15 s after an in-line solenoid valve was opened. Nitrogen dioxide and nitrogen monoxide (NO) concentrations of the test gases were measured with a dual beam IR-UV spectrophotometer (Binos, Inficon Leybold-Heraeus, Inc., Germany) and confirmed with a manual detector (model 8014–400A, Matheson Gas Products, East Rutherford, NJ). The NO_2 dose presented to the animal was calculated as the product of gas concentration (mg/l), inspired minute ventilation (l/min) and exposure duration (min), and normalized to body weight (kg).

2.8. Data analysis

All statistical comparisons were conducted on raw data. A one-way and two-way analysis of variance (ANOVA) was performed for effects of gas concentration and effects over time, respectively. If significance was found,

the ANOVA was followed by a Duncan's multiple-range test to determine where the significant differences existed. A Mann-Whitney Test was used to compare the nose-only and lung-only groups for the NO_2 dose and mean inspired minute ventilation (during exposure) variables. Significance was accepted at the 95% ($P < 0.05$) confidence level. Data, unless otherwise noted, are expressed as the mean ± S.D., with ranges shown in parentheses.

3. Experimental protocols

3.1. Nose-only vs. lung-only exposures

Pulmonary mechanics, hemodynamics and respiration were measured and blood samples obtained immediately pre- (baseline) and post exposure, and at 1, 4 (Day 1) and 24 h (Day 2) post exposure. Inspired minute ventilation was also measured during exposure. The nasotracheal tube was removed between the 4- and 24-h sampling times and re-introduced for 24 h post exposure sampling. In addition, the nasotracheal tube was removed 1 h prior to exposure and re-inserted immediately post exposure for nose-only experiments.

3.2. Bronchoalveolar lavage experiments

Cardiopulmonary function was monitored and blood samples obtained 2 weeks prior to exposure, immediately pre- and post exposure, and at 6 (Day 1) and 24 h (Day 2) post exposure. Inspired minute ventilation was also measured during exposure. The nasotracheal tube was removed between the 6- and 24-h sampling times and re-introduced for 24-h post-exposure sampling. Bronchoalveolar lavages were performed after cardiopulmonary measurements and blood collection at 2 weeks prior to exposure (baseline, −2 weeks), immediately post exposure, and at 6 and 24 h post exposure. The temporal sequence of lavage sampling was conducted in this manner to reduce effects of the BAL procedure, per se, on pulmonary mechanics measurements.

4. Results

4.1. Nose-only vs. lung-only exposures

The mean NO_2 dose inhaled by sheep was 7.1 ± 2.1 (4.3–11.0) mg/kg for the lung-only group, while that for the nose-only group was 4.5 ± 2.2 (2.1–7.4) mg/kg ($P < 0.05$). Nitric oxide concentrations in the test gases were negligible.

No significant changes were detected in arterial or mixed-venous hematocrit, total hemoglobin, carbon dioxide partial pressure, plasma bicarbonate or heart rate, oxygen consumption and hematologic variables

between exposure groups. Some fluctuations in arterial and mixed-venous pH were observed between exposure groups and among sampling times but all values were within normal ranges. Statistically significant 1.0 and 1.5% decreases in baseline core temperature (39.2 and 39.5°C) occurred at 4 h post exposure in the nose-only and lung-only groups, respectively. However, these effects were not significantly different from each other between groups at the same sampling time and core temperature in both groups were similar to pre-exposure values 24 h after exposure.

Nitrogen dioxide, delivered through the nasotracheal tube (lung-only), caused an immediate 64% ($P < 0.05$) increase in mean inspired minute ventilation (Table 1). The increase resulted from a 129% ($P < 0.05$) breathing rate increase, despite a 20% (n.s.) decrease in mean tidal volume. The increases in inspired minute ventilation and breathing rate remained significantly elevated at 1 h post exposure. These respiratory changes returned towards baseline within the 24 h post-exposure period. When delivered through a face mask (nose-only), NO_2 caused no appreciable tidal volume change, and smaller increases in breathing rate and inspired minute ventilation were observed early after exposure, compared to lung-only group values. Significant R_L and C_{dyn} changes were observed in both nose-only and lung-only groups after exposure (Fig. 1 and 2). Lung resistance increased progressively within the 24-h period following exposure in the lung-only group and R_L values were significantly elevated over baseline and control at both 4 and 24 h post exposure. Dynamic lung compliance in the lung-only group decreased to approximately 50% of baseline immediately post exposure and remained at that approximate level for the remainder of sampling times within the 24-h post-exposure sampling period. In the nose-only group, R_L remained within normal range through the 1-h sampling time. Lung resistance increased 25% (n.s.) above baseline at 4 h post exposure and 93% ($P < 0.05$) above baseline at 24 h post exposure. Dynamic lung compliance in the nose-only group remained within normal range through the 4-h sampling time but decreased to 63% (n.s.) of baseline at 24 h post exposure.

Nitrogen dioxide effects on cardiovascular variables and hemodynamics were minimal and essentially limited to the pulmonary circulation (Table 2). In the nose-only group, both pulmonary artery pressure and pulmonary artery wedge pressure remained within normal ranges throughout the 24-h post-exposure sampling period. In the lung-only group, both pulmonary artery pressure and pulmonary artery wedge pressure values were within normal ranges through 4 h post exposure. However, both hemodynamic variables were significantly elevated above baseline at 24 h post exposure. The pulmonary vascular pressure changes did not significantly affect resistance through the pulmonary vascular bed. Cardiac output decreased significantly at all sampling times post exposure during the first experimental day in the

Table 1
Effect of 15-min, 500-ppm NO_2 on selected respiratory and blood chemistry variables; nose-only (NO) vs. lung-only (LO) exposures

Variable	Treatment	Sampling time				
		Pre	Post	+1 h	+4 h	+24 h
Tidal volume	NO	309 (77)	313 (132)	371 (91)	361 (84)	305 (58)
(ml)	LO	304 (99)	242 (88)	290 (125)	284 (95)	330 (60)
Inspired minute ventilation	NO	6.8 (1.8)	8.5 (3.5)	8.2 (2.3)	7.0 (1.3)	10.0 (5.0)
(l/min)	LO	9.5 (2.0)	15.6 (4.0)[a,b]	17.1 (5.3)[a,b]	9.5 (7.3)	10.6 (3.6)
Breathing rate	NO	24 (10)	35 (27)	24 (10)	21 (7)	38 (28)
(breaths/min)	LO	28 (10)	64 (20)[a]	62 (28)[a,b]	30 (25)	30 (13)
Arterial PO_2	NO	97.5 (8.5)	95.9 (5.7)	94.7 (2.7)	93.9 (7.6)	89.6 (10.2)
(Torr)	LO	91.2 (7.9)	95.5 (7.2)	88.1 (6.8)	88.7 (14.6)	67.1 (11.0)[a,b]
Arterial PCO_2	NO	33.7 (2.1)	36.4 (3.5)	34.9 (2.1)	35.6 (3.4)	32.3 (3.2)
(Torr)	LO	32.6 (3.2)	30.7 (3.4)	30.8 (2.6)	33.9 (2.1)	30.4 (1.9)
[Methemoglobin]	NO	1.4 (0.2)	2.7 (0.4)[a]	1.9 (0.3)[a]	1.4 (0.1)	1.4 (0.2)
(%)	LO	1.2 (0.1)	4.0 (1.5)[a,b]	2.8 (1.2)[a,b]	1.5 (0.4)	1.2 (0.4)

Note: Data are mean (standard deviation), $n = 5–6$.
[a] $P < 0.05$, compared to baseline (pre-exposure).
[b] $P < 0.05$, compared to each other at the same sampling time.

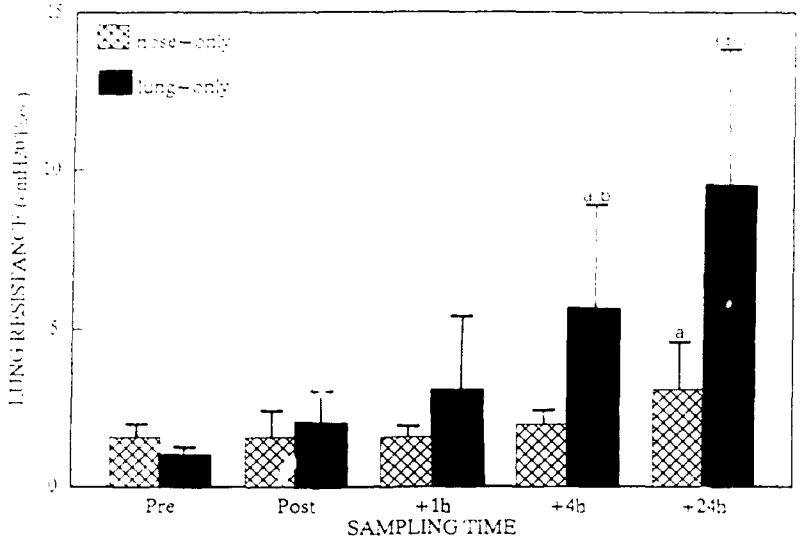

Fig. 1. Effect of 15-min, 500-ppm NO_2 exposure on lung resistance. Data are the mean ± S.D. for 5–6 sheep. $^{a}P < 0.05$, compared to baseline (pre-exposure); $^{b}P < 0.05$, compared to each other at the same sampling time; $^{c}P < 0.05$, compared to all other sampling times in the exposure group.

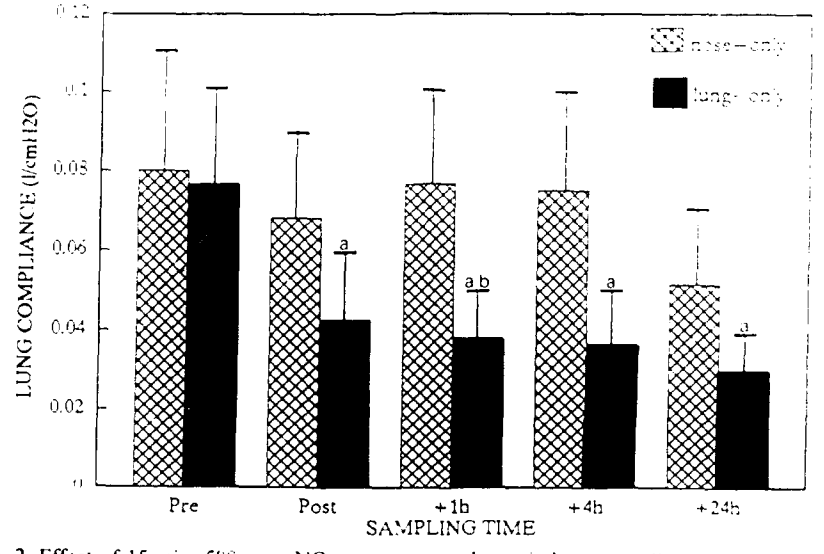

Fig. 2. Effect of 15-min, 500-ppm NO_2 exposure on dynamic lung compliance. Data are the mean ± S.D. for 5–6 sheep. $^{a}P < 0.05$, compared to baseline (pre-exposure); $^{b}P < 0.05$, compared to each other at the same sampling time.

Table 2
Effect of 15-min, 500-ppm NO_2 on pulmonary hemodynamics variables; nose-only (NO) vs. lung-only (LO) expos...

Variable	Treatment	Sampling time				
		Pre	Post	+1 h	+4 h	+24 h
Pulmonary artery pressure	NO	15.6 (2.8)	15.1 (4.3)	15.4 (4.0)	15.8 (6.4)	17.3 (2.1)
(cmH_2O)	LO	17.4 (3.6)	18.2 (3.1)	18.2 (4.6)	17.1 (4.1)	24.5 (4.0)[a,b]
Pulmonary artery wedge	NO	6.0 (3.0)	6.3 (4.1)	5.4 (2.4)	5.8 (6.4)	6.3 (3.1)
pressure (cmH_2O)	LO	6.3 (2.4)	4.8 (2.9)	5.6 (3.1)	4.9 (2.7)	11.1 (2.3)[a,b]
Pulmonary vascular resistance	NO	1.8 (0.2)	2.2 (0.8)	2.3 (0.4)	2.4 (0.5)	1.9 (0.4)
(cmH_2O/l/min)	LO	1.8 (0.2)	2.3 (0.5)	2.4 (0.7)	3.0 (1.1)[a]	2.4 (0.6)
Cardiac output	NO	5.6 (0.6)	4.3 (0.8)[a,b]	4.4 (1.0)[a]	4.3 (1.2)[a]	6.2 (0.8)
(l/min)	LO	6.2 (1.3)	5.9 (0.8)	5.5 (1.3)	[illegible].4 (1.3)[a]	5.6 (1.8)

Note: Data are mean (standard deviation), $n = 5–6$.
[a] $P < 0.05$, compared to baseline (pre-exposure).
[b] $P < 0.05$, compared to each other at the same sampling time.

nose-only group, whereas a significant cardiac output decrease in the lung-only group was noted only at 4 h post exposure.

Histopathologic examination of tissue from lung-only exposed sheep revealed exudative fluid distributed in a patchy lobular pattern, focally disseminated and equally distributed in all lobes. Mild neutrophil infiltration was found in alveolar sacs and interalveolar capillaries, particularly in areas of fluid exudation. Examination of lung tissue from nose-only NO_2-exposed sheep revealed little evidence of exudation. However, an increased number of leukocytes was observed in interalveolar capillaries (compared to air exposed sheep).

4.2. Bronchoalveolar lavage experiments

The mean dose inhaled by sheep in the NO_2 group was 6.0 ± 2.0 (2.7–9.1) mg/kg. No NO_2 was detected in the control (air) test gas. Nitric oxide was not detected in either the air or NO_2 test gases.

No statistical differences were found in all variables examined either between groups or within groups at the −2 week and pre-exposure sampling times. Negligible lung mechanics, respiratory, biochemical or lung pathologic changes occurred in air exposed sheep. However, a 15% (n.s.) cardiac output decrease and 1.8% ($P < 0.05$) core temperature decrease occurred at the 4-h sampling time. Since the pathophysiologic profile of sheep given 500 ppm NO_2 via nasotracheal tube has been previously described ((Januszkiewicz et al., 1992); Figs. 1 and 2; Tables 1 and 2), only a limited number of variables which typify the high-level exposure are shown (Table 3). Mean inspired minute ventilation during the NO_2 exposure was 13.4 ± 4.8 l/min. Early post-exposure respiration was characterized by an 86% (n.s.) increase in breathing rate and a 35% ($P < 0.05$) tidal volume decrease, compared to pre-exposure baseline. Within the 24-h sampling period, respiration had approached the pre-exposure pattern. Mean R_L remained within normal limits immediately after NO_2 exposure (Table 3). Lung resistance increased 94% at 6 h and 227% above baseline at 24 h post exposure. Dynamic lung compliance decreased progressively following NO_2 exposure to the lowest value at 24 h post exposure. Arterial oxygen partial pressure changes were negligible during the first 6 h following exposure. However, a significant decrease in arterial oxygen occurred at 24 h post exposure. The NO_2 exposure was marked by a transient 333% ($P < 0.05$, compared to baseline) blood methemoglobin increase, which returned to baseline by the 6-h sampling time.

Nitrogen dioxide effects on the cardiovascular system were minimal. A 15% (n.s.) pulmonary artery pressure increase occurred at 24 h post exposure. Both pulmonary artery wedge pressure and pulmonary vascular resistance remained relatively constant in the post-exposure sampling period,

Table 3
Effect of 20-min exposures to either air (0) or 500 ppm NO_2 on selected pulmonary mechanics and blood chemistry variables

Variable	$[NO_2]$ (ppm)	Sampling time				
		−2 weeks	Pre	Post	+6 h	+24 h
Lung resistance	0	1.5 (0.5)	1.6 (0.4)	2.3 (1.2)	1.8 (1.1)	1.5 (0.3)
($cmH_2O/l/s$)	500	1.3 (0.7)	1.8 (1.0)	1.6 (0.6)	3.5 (2.2)	5.9 (2.4)[a,b]
Dynamic lung compliance	0	60.3 (8.8)	60.2 (18.3)	56.5 (15.2)	71.3 (14.0)	96.0 (49.7)
(ml/cmH_2O)	500	64.2 (17.2)	51.7 (8.7)	48.7 (7.5)	38.8 (12.0)	30.7 (8.0)[a,b]
Arterial PO_2	0	100.2 (10.3)	101.5 (3.3)	104.0 (1.5)	103.4 (8.7)	101.3 (8.9)
(Torr)	500	98.1 (11.0)	100.2 (8.3)	92.0 (10.0)	89.0 (7.7)	65.2 (13.7)[a,b]
[Methemoglobin]	0	0.8 (0.1)	0.8 (0.2)	1.0 (0.3)	0.8 (0.2)	0.9 (0.4)
(%)	500	0.8 (0.3)	0.9 (0.1)	3.9 (0.9)[a,b]	0.7 (0.0)	0.6 (0.1)

Note: Data are mean (standard deviation), $n = 5–6$.
[a]$P < 0.05$, compared to baseline (pre-exposure).
[b]$P < 0.05$, compared to control (air-exposed) at the same sampling time.

compared to baseline values (8.9 cm H_2O and 2.1 cm H_2O/l/min, respectively).

The BAL procedure was well-tolerated by conscious sheep. Average BALF return was 74 ± 4% for combined air and NO_2 baseline measurements. An average 151-ml BALF return was achieved for both air and NO_2 groups immediately post exposure (104 and 99% of baseline, respectively). Subsequent BALF returns decreased to 89% of baseline for air-exposed sheep. Bronchoalveolar lavage fluid return decreased to 65% of baseline ($P < 0.05$) at 6 h post exposure and increased to 82% of baseline at 24 h post exposure for NO_2-exposed sheep. No significant change in BALF total cell count was found either between exposure groups or among the different sampling times. Total cell count over all sampling times and exposure groups averaged 14.4×10^6 cells. Less than 0.25% of the cells could not be identified as to type. Few epithelial cells were found in BALF from air-exposed sheep or baseline BALF in the NO_2 group (Table 4). However, the BALF epithelial cell number increased significantly at all sampling times after NO_2 exposure. At 6 and 24 h post exposure, the epithelial cells were degenerative and shrunken with irregular cell margins, deeply eosinophilic nuclei, and dark basophilic cytoplasm. Air exposure caused negligible change in BALF protein content over time (Fig. 3). Conversely, NO_2 exposure caused significant increases in BALF protein at 6 and 24 h post exposure. Nitrogen dioxide also caused significant decreases in BALF macrophage count at all sampling times following exposure (Fig. 4). Changes in BALF protein, and epithelial and macrophage count were largest at 6 h post exposure (Table 4, Figs. 3 and 4).

Histopathologic examination of tissue from NO_2-exposed sheep revealed a similar injury pattern to that previously described for lungs from sheep exposed to 500 ppm NO_2 through a nasotracheal tube (Januszkiewicz et al.,

Table 4
Effect of 20-min exposures to either air (0) or 500 ppm NO_2 on BALF epithelial cell number (% of BALF total cell count)

	Sampling time			
	−2 weeks	Post	+6 h	+24 h
[NO_2] (ppm)				
0	0.1 (0.3)	0.3 (0.5)	0.1 (0.3)	0.2 (0.4)
500	0.6 (0.5)	14.3 (15.2)[a,b]	36.0 (13.0)[a,b]	21.0 (16.0)[a,b]

Note: Data are mean (standard deviation), $n = 5–6$.
[a] $P < 0.05$, compared to baseline (−2 weeks).
[b] $P < 0.05$, compared to control (air-exposed) at the same sampling time.

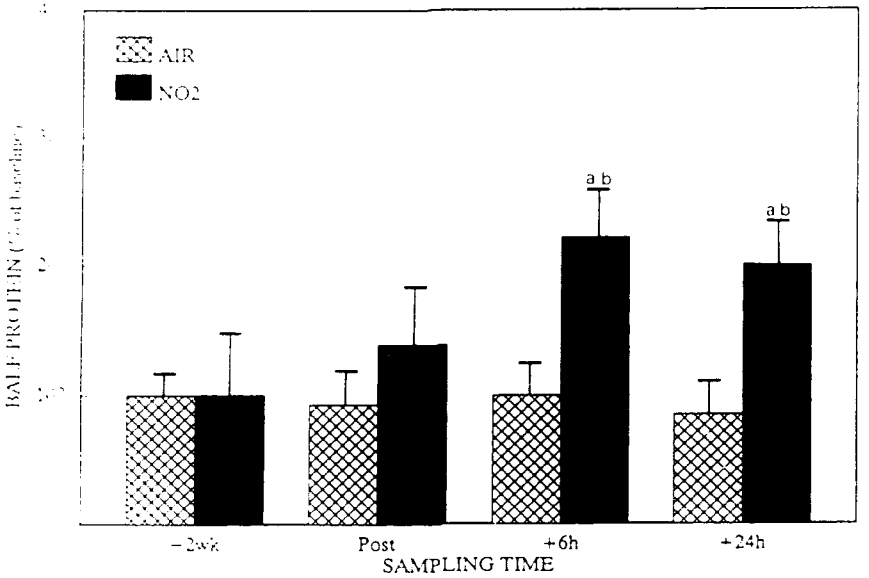

Fig. 3. Effect of air or 500 ppm NO_2 exposure on bronchoalveolar lavage protein concentration, expressed as percent of the baseline measurement (−2 weeks). Data are the mean ± S.D. for 5–6 sheep. $^{a}P < 0.05$, compared to control (air-exposed); $^{b}P < 0.05$, compared to baseline BALF (−2 weeks).

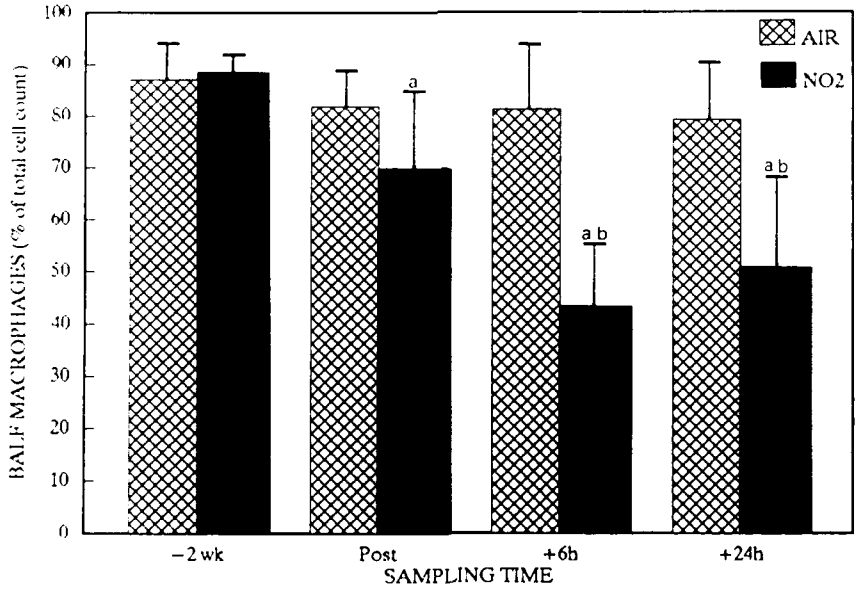

Fig. 4. Effect of air or NO_2 exposure on bronchoalveolar lavage macrophage number, expressed as percent of the BALF total cell count. Data are the mean ± standard deviation for 5–6 sheep. $^{a}P < 0.05$, compared to control (air-exposed); $^{b}P < 0.05$, compared to baseline BALF (−2 weeks).

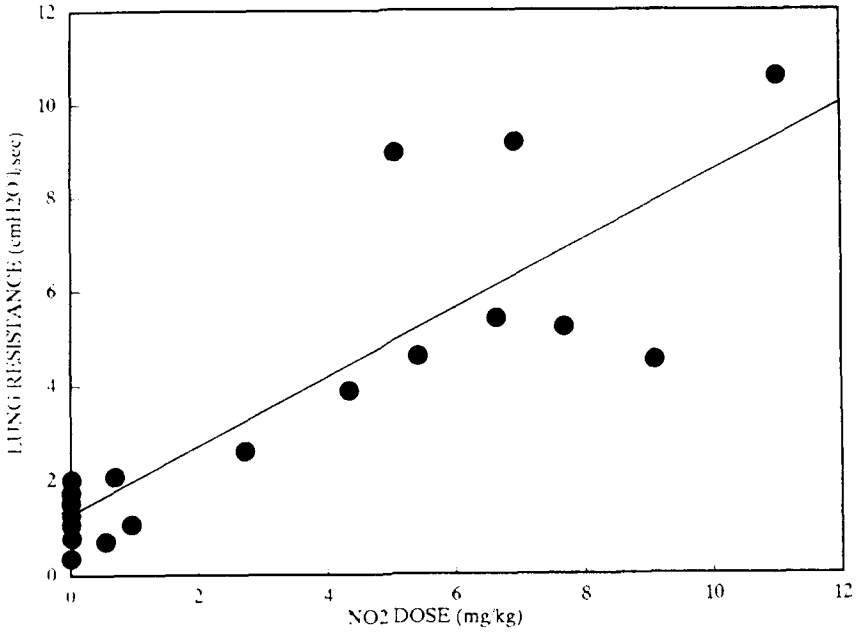

Fig. 5. Relationship between lung resistance (measurement at 24 h post exposure) and NO_2 dose presented to the animal. Data represent individual measurements from 22 sheep exposed to either air, 100 ppm NO_2 for 15 min (Januszkiewicz et al., 1992) or 500 ppm NO_2 for 15–20 min. The correlation coefficient for the dose-response relationship was 0.87 ($P < 0.05$).

1992). A patchy-lobular pattern of eosinophilic-stained fluid was distributed among all lobes. The number of neutrophils and mononuclear leukocytes was increased, compared to control, and damage was generally greater in terminal bronchioles, alveolar ducts and alveoli. No lesions characteristic of NO_2 toxicity were observed in lung tissue from air-exposed sheep.

4.3. Dose — response relationships

The NO_2 dose presented to the sheep was plotted against the 24-h post-exposure R_L measurement (Fig. 5). Data represent individual measurements from 22 animals in these and previous experiments (Januszkiewicz et al., 1992), where sheep were exposed through a nasotracheal tube to either medical-grade air, 100 ppm NO_2 for 15 min, or 500 ppm NO_2 for either 15 or 20 min. The correlation coefficient between variables was 0.87 ($P < 0.05$).

5. Discussion

These experiments showed that short-duration exposure to high-concentration NO_2 produced pronounced lung effects in the adult awake

sheep. Moreover, a consistent toxicologic profile appeared. Five-hundred ppm NO_2 administered directly into the lungs of sheep was marked by a significant, albeit small, blood methemoglobin increase. The exposure induced an immediate tachypnea, resulting in an overall minute ventilation increase. Within 1 day following exposure, pulmonary function progressively deteriorated, and signs suggesting pulmonary exudation appeared. Maximal lung mechanics effects were associated with significant hypoxemia 24 h after insult.

Histopathologic examination 24 h after exposure revealed patchy, focally-disseminated exudative material in all lung lobes and a small neutrophil influx. Information from BALF supported pathological findings of lung injury and suggested epithelial damage and transvascular protein flux. Nitrogen dioxide-induced endothelial cell tight junction alterations (Gordon et al., 1986), protein transudation (Gurley et al., 1989), and increased BALF epithelial cell number (Man et al., 1990) have been reported for hamsters, rats and dogs, respectively.

Hypoxemia-induced pulmonary hypertension (Marshall and Marshall, 1983) was anticipated following NO_2 exposure. However, maximal pulmonary artery pressure increases after exposure were mild and no significant pulmonary vascular resistance change occurred. One reason for the apparent lack of hemodynamic effects despite severe pulmonary involvement could be related to NO_2 breakdown products. Frostell et al. (1991) reported that 80 ppm nitric oxide reversed acute pulmonary vasoconstriction in sheep without systemic vasodilation, methemoglobin formation, or lung histology changes. Although the toxicodynamics of high-concentration NO_2 are unresolved, perhaps residual nitrogenous breakdown products of NO_2 could be responsible for this phenomenon.

The terms nose-only and lung-only refer to the primary route of exposure and technology used to deliver the test gases (face mask or nasotracheal tube), not end-organs affected. Although nose-only and lung-only exposures are not synonymous with quiet-normal breathing and mouth breathing, respectively, they provide similarity to these respiratory patterns. While exposure by face mask allows the test gas to traverse the nasal passages before entering the lung, intubation bypasses the upper airways. The two routes of exposure produced NO_2 toxicologic profiles significantly different from each other. While lung-only NO_2 exposures significantly increased respiration and caused edema and hypoxemia, nose-only exposures did not. In addition, lung-only pulmonary mechanics changes were significantly different compared to corresponding values from the nose-only group. The mechanism for the attenuated response in nose-only NO_2-exposed sheep was not systematically studied. However, NO_2 scrubbing through the nasal passages is a plausible explanation. Nitrogen dioxide could be removed by absorption onto the large surface area of the sheep nasal cavity, react with water during

the humidification process, or possibly interact with nasal antioxidants (Peden et al., 1990). Hypothetically, these processes would lead to a decreased NO_2 fraction that could reach tracheobronchial regions and produce a stimulatory effect on respiration. An attenuated inspired minute ventilation increase would ultimately result in a decreased NO_2 dose presented to the animal. This hypothesis is supported by the observation that although the measured NO_2 concentrations between nose-only and lung-only groups were similar, both the inspired minute ventilation increase and NO_2 dose were significantly reduced in the nose-only group. Unpublished results from our laboratory showed that a large proportion (48.5 ± 4.4%, $n = 4$) of inspired NO_2 (521 ± 13 ppm) was removed when sheep breathed the gas through a face mask (combined nasal and pulmonary removal).

The protective effect afforded by the nasal passages is a factor which dynamically decreased both dose and response of a specific NO_2 concentration (C) and exposure duration (T, time). Numerous other factors can influence the dose of inhaled substances. These dosing factors include uptake, regional deposition, metabolism and clearance processes, in addition to breathing patterns. Inspired minute ventilation, or accumulative volume of inspired gas per unit of time, was a useful dose determinant examined in these experiments. The response to a 500 ppm NO_2 lung-only exposure was more severe than the response following a nose-only exposure, based on blood gas, lung mechanics alterations and pathology findings. Moreover, the NO_2 dose presented to the sheep in the nose-only group was approximately 63% ($P < 0.05$) of the dose for sheep in the lung-only group. Similarly, the NO_2 dose received by sheep in the BAL experiments after 500 ppm NO_2 was approximately 85% of the NO_2 dose received by sheep in the lung-only group. Correspondingly, maximal R_L increases in BAL NO_2-exposed sheep were smaller and post-exposure C_{dyn} decreases were more gradual, compared to the precipitous, immediate and sustained C_{dyn} decrease in the lung-only group. The importance of inspired minute ventilation as a dose determinant is clearly demonstrated (Fig. 5). Lung resistance was selected as a toxic endpoint since it appeared to be the most sensitive indicator of insult, compared to blood gas or pathology variables. Furthermore, the NO_2-induced tachypneic breathing pattern was expected to diminish deep lung injury at the expense of the conducting airways (DuBois, 1968) and the R_L measurement, in part, reflected upper airway status.

Haber's Law ($C \times T = K$, where K is a constant) conveys the importance of cumulative dose in the expression of a toxic event. Whether NO_2 follows Haber's Law is uncertain. Some reports suggested that dose rate, rather than cumulative dose, was a better predictor of NO_2 poisoning (Gardner et al., 1979; Stavert and Lehnert, 1988). Others concluded that, with respect to NO_2, Haber's Law was partially dose rate-dependent (Gelzleichter et al.,

1992). The *C*- or *T*-dependency of NO_2 toxicity could not be determined from results obtained from our sheep experiments because of the limited number of gas concentrations and exposure durations examined. However, a strong linear relationship between NO_2 dose and the lung resistance response was demonstrated. These data suggest that the NO_2 cumulative dose may indeed be important to the NO_2 response, when fundamental physiologic dose-determinants are applied. Studies that have systematically examined NO_2 and Haber's Law have not addressed NO_2-induced breathing pattern changes during exposure, which ultimately affect dose. Perhaps *C*-dependant shifts in respiratory pattern during exposure could explain NO_2's apparent non-conformity with Haber's Law.

Accurate dose measurements are generally not available for accidental exposures to NO_2. However, on-board equipment afforded some insight into an inadvertent NO_2 exposure of the crew on a manned space mission (Hatton et al., 1977). Three Apollo-Soyuz astronauts were accidently exposed to an estimated 250 ppm NO_2 (peak, 750 ppm) for approximately 4.7 min. Crewmembers were able to complete the mission, although immediate breathing difficulties were reported, and an average 4.2% methemoglobin level was determined upon re-entry. Respiratory difficulties became more severe within 24 h following exposure and chest radiographs suggested diffuse chemical pneumonitis. Several days after exposure the astronauts became asymptomatic and chest radiographs were normal. The non-lethal effects and rapid recovery from the massive exposure suggested that the NO_2 dose ($C \times T$) received by the astronauts was overestimated. However, it is also possible that the toxic response to that exposure may have been overestimated, based on lethal predictions for humans which consider inspired minute ventilation as a dose scaling factor (Book, 1982). Extrapolation of experimental animal results to humans and risk criteria development are complex issues. Furthermore, results of high-concentration NO_2 in sheep cannot be directly applied to humans, particularly for nose-only exposures where substantial nasal volume differences exist between species. However, sheep data support the concepts of a generalized mammalian response and size-dependant species sensitivity to NO_2. While sheep (Januszkiewicz et al., 1992) and dogs (Man et al., 1990) have been shown to survive exposures up to 500 ppm for 15–20 min and 400 ppm for 1 h, respectively, these exposures would likely be immediately lethal to smaller-sized animals. For example, Johnson et al. (1982) exposed dogs to 69 ppm NO_2 for 6 h and reported a 'lack of significant pulmonary edema'. Conversely, Hine et al. (1970) reported a 58–100% rat mortality following exposure to 75 ppm NO_2 for 4–8 h, respectively. As another example, while exposure to 100 ppm NO_2 for 15 min produced significant lung gravimetric changes in rats (Stavert and Lehnert, 1990), the same $C \times T$ NO_2 exposure caused

negligible physiologic effects or demonstrable pathological lesions in sheep (Januszkiewicz et al., 1992). Fundamental differences in respiratory physiology among species (Book, 1982; Phalen, 1984) may explain the general observation that small animals appear more sensitive to NO_2 than large animals.

Salient features of pulmonary NO_2 toxicity in sheep include severe hypoxemia, relatively non-compliant lungs, inflammatory cell influx, pulmonary infiltrates (Januszkiewicz et al., 1992) and increased airway responsiveness (Abraham et al., 1980b). Perhaps NO_2 insult in this species may have applicability as an experimental animal model for the adult respiratory distress syndrome (Bachofen et al., 1979). Dissimilar to some models of acute lung injury (Simpson et al., 1991), insult occurs subsequent to inhalation exposure. Non-cardiogenic pulmonary edema is produced by brief exposure and unlike hyperoxia (Newman et al., 1983; Fukushima et al., 1990) widespread manifestations of poisoning are observed within 24 h after insult. Sheep are commonly used for lung fluid balance studies. Furthermore, our experiments with sheep showed that negligible hemodynamic effects occurred after high-level NO_2 exposure — a desirable phenomenon when fluid dynamics assessments are made during the development of permeability edema.

High-concentration NO_2 exposure continues to be an important concern in the civilian and military workplaces. Previous sheep experiments focused on the NO_2 threshold for significant vital function abnormalities (Januszkiewicz et al., 1992). The experiments described here addressed administration route effects on NO_2 pathophysiology. Furthermore, a fundamental dose determinant was assessed. In addition, preliminary analysis of BALF from NO_2-exposed sheep revealed the appearance of endogenous inflammatory mediators (Fitzpatrick et al., 1993). Experiments are currently underway to investigate NO_2 effects on nasal structure and function, and the sheep exercise model (Dodd et al., 1992) is being used to assess NO_2-induced effects on physical performance. Both anecdotal reports of human accidental exposure and scientifically-controlled animal studies have played an important role in understanding NO_2 pathogenesis. Experiments which address underlying injury mechanisms for this noxious agent may ultimately lead to new preventative measures and therapeutic regimens.

6. Acknowledgements

The authors wish to thank Mr. Harold F. Summey, Specialist Randy O. Willmott and Sergeant Jonathan W. Sturgis for technical support and Barbara J. Januszkiewicz for artwork. We also gratefully acknowledge Drs. Nicholas M. Coulson, Michael S. Lagutchik and Carol A. Bossone for veterinary assistance, Douglas D. Sharpnack and William Inskeep II for pathological evaluations and Daniel B. Rayburn for technical advice.

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